Aquaculture 324–325 (2012) 158–164

Contents lists available at SciVerse ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Sublethal effects of alizarin complexone marking on Baltic cod (*Gadus morhua*) eggs and larvae

Stefan Meyer [a,*], Sune R. Sørensen [b], Myron A. Peck [a], Josianne G. Støttrup [b]

[a] Institute for Hydrobiology and Fisheries Science, University of Hamburg, Olbersweg 24, 22767, Hamburg, Germany
[b] Section for Coastal Ecology, National Institute of Aquatic Resources (DTU Aqua), Technical University of Denmark, Charlottenlund Castle, DK-2920 Charlottenlund, Denmark

ARTICLE INFO

Article history:
Received 14 March 2011
Received in revised form 19 September 2011
Accepted 25 October 2011
Available online 7 November 2011

Keywords:
Baltic cod
Alizarin complexone
Batch marking
Chronic effects
Stock enhancement

ABSTRACT

Standard, single-exposure alizarin complexone- (ALC) marking was conducted on early life stages of Baltic cod (*Gadus morhua* L.) to examine acute and chronic effects. Embryos and yolk sac larvae were marked using different concentrations of ALC (0, 50, 100 and 200 mg l^{-1}). Experiments included control groups for treatment and handling effects. In agreement with previous studies, long-lasting, distinct otolith marks were produced by immersion of yolk sac larvae in ≥50 mg l^{-1} ALC for 24 h. Mortality of eggs and larvae was low during the marking procedure. Hatching success of ALC marked embryos was significantly reduced and hatching was delayed with increasing ALC concentration. Growth rates of larvae through 21 *dph* (exogenous feeding stage) were significantly reduced in ALC marked fish compared to controls. Biochemical condition (RNA–DNA ratio) was not affected. Subtle changes in activity and metabolism were indicated by reduced first feeding success and yolk absorption rates. Our results reveal that batch marking of finfish early life stages using ALC can have not only significant, acute impacts on survival but, despite long-standing assumptions to the contrary, also sublethal effects on other vital parameters like growth. We discuss these acute and chronic effects and give recommendations for assessment of life stage- and species-specific ALC immersion marking procedures.

1. Introduction

Marking and identification techniques are an integral part of responsible stock enhancement and restocking programs (Blankenship and Leber, 1995; Lorenzen et al., 2010) and have been tested and employed in marine finfish and invertebrate species worldwide (e.g., Liu et al., 2009; Purcell and Blockmans, 2009). Immersion of finfish eggs, larvae or juveniles in chemical compounds such as alizarin- and oxytetracyclin-derivates that form fluorescent active complexes with calcified structures has proven to be cost effective and easily applied to large numbers of individuals (Babaluk and Craig, 1990; Nagięć et al., 1995; Pedersen and Carlsen, 1991; Rojas Beltran et al., 1995). Alizarin complexone (ALC) yields a distinct, long-lasting mark on otoliths, allowing reliable identification of specimens captured after release and has been applied on a number of marine fish species including Atlantic cod (*Gadus morhua*), turbot (*Psetta maxima*) and gilthead sea bream (*Sparus aurata*) (e.g., Blom et al., 1994; Sanchez-Lamadrid, 2001; Støttrup et al., 2002).

Although most studies optimizing ALC marking protocols for stock enhancement of finfish and aquatic invertebrates only measure survival as a proximate endpoint, the ultimate conclusion drawn from the literature is that ALC has no persistent adverse effect on the marked organism (Blom et al., 1994; Tsukamoto, 1988; Tsukamoto et al., 1989). Some evidence supports this assertion when ALC is used in older, juvenile finfish (e.g., Baumann et al., 2005; Liu et al., 2009), but few studies have systematically examined the potential persistent impacts of ALC marking. This is surprising since these sublethal effects could seriously undermine stocking success. Measurements of rates of metabolism and somatic growth, biochemical indices of condition (e.g., RNA–DNA ratio) and behavioral characteristics are known to reflect the overall condition of marine fish larvae (Ferron and Leggett, 1994), yield a function-based understanding of fish welfare (Fraser, 1999; Huntingford et al., 2006) and would provide useful indications of sublethal, chronic effects. Furthermore, the interplay of these proximate measures can provide insight on the ultimate, underlying pathophysiological processes caused by ALC exposure.

The aim of the present study was to establish ALC marking protocols for embryos and yolk sac larvae of Baltic cod and to investigate how marking and handling procedures affected these early life stages. Proximate effect parameters were assessed on a continuum from acute (survival, hatching) to chronic (yolk absorption, growth rates, RNA–DNA ratio, and feeding incidence). This research was conducted as part of a project aimed at restocking Eastern Baltic cod by releasing first feeding yolk-sac larvae (Støttrup et al., 2008a). Therefore, the priority of the present research was to develop techniques that had the lowest possible impacts on the survival, growth and condition of cod pre-recruit life stages.

* Corresponding author at: Association for Marine Aquaculture, Hafentörn 3, 25761 Büsum, Germany. Tel.: +49 4834 96539915; fax: +49 4834 96539999.
E-mail address: meyer@gma-buesum.de (S. Meyer).

0044-8486/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquaculture.2011.10.041

2. Material and methods

2.1. General experimental conditions

Baltic cod eggs and larvae were produced by broodstock fish caught in the eastern Baltic that were maintained at a commercial cod hatchery (Bornholm's Lakseklækkeri, Nexø, Bornholm, Denmark) for at least 6 months prior to spawning. Eggs were collected from spontaneous group spawning, disinfected and incubated in slightly aerated, flow-through (20 l h^{-1}), conical 80 l incubators, using methods described by Støttrup et al. (2008a), until the start of experiments.

Marking procedures described below used aqueous solutions of alizarin complexone (ALC; CAS-number: 3952-78-1; buffered with 1 M KOH) prepared in artificial saltwater immediately prior to the start of the marking procedure. During the 24 h marking procedure, salinity (year 2006, 19 ± 0.1 psu, mean ± range; year 2008, 15 ± 0.1 psu) and temperature (7 ± 0.2 °C) were similar to incubation conditions. High oxygen saturation (93 ± 5.0%) levels and stable pH (8.3 ± 0.3) were maintained. Given are means and ranges, respectively.

2.2. Effect of ALC concentration

A first experiment investigated the influence of different ALC concentrations on embryo survival and hatching success (year 2006). Eggs spawned on July 2nd were removed 1 day prior to expected hatching and immersed for 24 h at either 0, 50, 100 or 200 mg l^{-1} ALC concentration in replicated (n = 2), slightly aerated 1 l marking containers each containing 110 eggs. After marking, eggs were transferred to 1.5 l beakers (n = 2) and were incubated for 7 days until all eggs were either hatched or embryos were dead. Hatched larvae were counted and removed each day.

Larval survival was assessed in early stage yolk sac larvae that were marked at 3 days post hatch (*dph;* spawned July 20th) using the same procedure as described above. Triplicate marking containers were used for each concentration and stocking density during marking was 50 larvae l^{-1}. After marking, larvae were loaded into 1.5 l beakers (n = 3) randomly arranged in a temperature controlled waterbath and were held for 9 days. Dead individuals were counted and removed each day.

2.3. Effect of ALC marking procedure

A second experiment investigated the handling-effect of ALC marking procedure on larval growth and condition (year 2008). Cod larvae used for this experiment stemmed from one spawning event (June 11th) and were (mean ± standard deviation, SD) 4.2 ± 0.2 mm standard length (*SL*), 60.1 ± 9.7 μg freeze-dried weight (*DW*) and had a yolk-sac area (*YSA*) of 1.46(±0.19) mm^2 at hatch. Yolk sac larvae (3 *dph*) were distributed among three treatment groups: a) larvae marked with 0 mg l^{-1} (handling group) or b) 50 mg l^{-1} ALC (marking group) in 25 l marking containers (100 larvae l^{-1} stocking density, 24 h exposure time, slightly aerated, 7 ± 0.2 °C, pH 8.3 ± 0.3, mean ± range) or c) larvae that were not manipulated in any way (control group). Control group larvae were left under pristine conditions in egg/larval incubators. After 24 h, 1500 larvae of each treatment group were divided evenly into 3 groups and transferred to triplicate 25 l rearing tanks (20 larvae l^{-1}). Water in the tanks was slightly aerated and mixed using airlift pumps separated from the larvae with a 320 μm mesh-sieve tank partition. Within 12 h after loading, water temperature was increased from 7.0 to 10.0 ± 0.2 °C (mean ± range). *Nannochloropsis* sp. (10^6 cells ml^{-1}) was added to each rearing tank and, from 5 dph, larvae were fed daily rations of calanoid copepod (*Acartia tonsa)* nauplii (> 1.5 ml^{-1}). Samples for larval growth and condition were taken from each tank every third day from 5 to 20 *dph*. At each sampling, 10 larvae were anesthetized with 50 mg l^{-1} MS222, photographed under a stereo microscope, rinsed with distilled water and stored at −80 °C prior to freeze-drying and RNA–DNA ratio (*RD*) analysis. Larval *SL* and *YSA* were measured with an image analysis software tool (ImageJ 1.41o, Wayne Rasband) to the nearest 0.1 mm and 0.1 mm^2, respectively, and *DW* to the nearest 0.1 μg (SE2 Ultra Micro, Sartorius). The growth experiment was terminated after 16 days (20 dph). The number of surviving larvae per tank was counted on this day.

Incidence of first feeding was assessed 24 h after marking (day 1 post exposure, *dpe*; 5 *dph*) using naïve larvae offered *A. tonsa* nauplii at a prey-concentration of 1 ml^{-1} for the first time. After 4 h feeding time, 10 fish were randomly sampled from each rearing tank and gut content was inspected using a stereo microscope.

Survival as a proximate consequence of marking procedure was assessed in yolk sac larvae that were transferred to 3 replicate survival tanks (50 larvae per tank) per treatment directly after marking. Tanks contained 4 l of slightly aerated water and no food was added. Larval survival (whole tank count) and morphometrics (*SL* and *YSA* of 20 larvae per tank) were assessed on 5 *dpe* (9 *dph*).

2.4. RNA–DNA ratio analysis

The RNA–DNA ratio of whole, individual larvae was analyzed using a fluorescent-dye, microplate assay, modified after Caldarone et al. (2001). In short, freeze-dried larvae were homogenized in 1% Sarcosil–Tris–EDTA-buffer using an ultrasonic disruptor, diluted with Tris–EDTA-buffer and two aliquots were mixed with Ethidium bromide (EB) in a 96-well plate. Fluorescence was measured at 520/605 nm (excitation/emission) in a microplate fluorometer (Xenius XC, SAFAS) and the average of the two aliquots was used. Subsequent addition of specific restriction enzymes (R 6513 and D 4263, Sigma Aldrich) eliminated RNA and DNA from the samples. Concentrations were determined based on calibration curves using highly-purified 18S + 28S rRNA from calf liver and calf thymus DNA (R 0889 and D 4764, Sigma Aldrich). A standardization factor ($SF_{Pi} = 0.33 \pm 0.05$, mean ± SE) for inter-laboratory comparison of RNA–DNA values was calculated based on the RNA and DNA standard curve slope ratios of all nucleic acid assays performed in this study (Caldarone et al., 2006).

2.5. Data analysis

The time of 50% maximum hatching success of eggs marked with different ALC concentrations was estimated by fitting third order polynomial regressions to tank-wise, daily cumulative hatching success data. Yolk absorption rates and *DW*-specific growth rates (% day^{-1}) were derived from the exponential regression model parameter g (instantaneous growth rate, day^{-1}) fitted to replicate tank-wise mean values versus time and calculated using the formula: $SGR = 100 * (e^g - 1)$. Absolute *SL*-growth rates (mm day^{-1}) were derived from linear regression models fitted to replicate tank-wise mean values of *SL* versus time. Percentage data were arcsine transformed [arcsine $(\%/100)^{0.5}$] prior to statistical testing. Treatment-related differences in *SL*- and *DW*-growth in exogenously feeding larvae (growth-experiment) were tested using Multivariate Analysis of Covariance (MANCOVA), using age (*dph*) as covariate. *DW* data were ln-transformed prior to MANCOVA. One-way ANOVA and subsequent post-hoc tests (SNK procedure) were used to test for differences in survival, hatching success, feeding incidence and yolk absorption rates. Kendall's W non-parametric test for concordance between ranks was used to discriminate treatment effects of ALC marking procedure across different effect parameters. Statistical significance was set at alpha = 0.05. If not stated otherwise, all means (± SE) are based on replicate tanks. All analyses were performed using SPSS (v18, SPSS Inc.).